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Rapid report

Negative influence of RasG on chemoattractant-induced ERK2 phosphorylation in *Dictyostelium*

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Abstract

The *Dictyostelium* ERK2 protein is transiently activated when cells are treated with the chemotactic agents cAMP or folic acid. Activating phosphorylation is markedly inhibited in strains overexpressing the constitutively activated RasG protein. This is in marked contrast to mammalian cells where the highly related mitogen-activated protein kinases (MAPKs) are stimulated by Ras activation. © 1998 Published by Elsevier Science B.V.

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The mitogen-activated protein kinases (MAPKs) play an important role in growth and development in mammalian cells (reviewed in Ref. [1]), and in addition are rapidly and transiently activated in response to chemotactic agents [2,3]. They are activated by phosphorylation on both the tyrosine and threonine residues of a TEY motif by a dual specificity kinase, MEK. Two MAPK homologues have been identified in *Dictyostelium discoideum* [4,5]. One of these, ERK2, is transiently activated in response to a pulse of extracellular cAMP [6] and cells with a disrupted *erk2* gene do not show activation of adenylyl cyclase in response to a pulse of cAMP and do not aggregate suggesting that ERK2 activation is intimately involved with signal relay [4]. This relay is essential for aggregation of cells in response to cAMP that occurs upon starvation in this organism.

The pathways leading to ERK2 activation in response to cAMP in *Dictyostelium* have been at least partially characterised. Examination of its activation in strains which are lacking components of the known signalling pathways suggests that the activation requires the interaction of extracellular cAMP with either of the cAMP receptors cAR1 or cAR3 [6,7]. It has been reported that activation is dependent on the Gα4 subunit [7]. However, this was determined using an immunoprecipitation kinase assay which may lack specificity (see below) and the result needs to be confirmed using a more direct assay. ERK2 is also transiently activated in response to another chemoattractant, folic acid [8,9], suggesting that its activa-

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tion plays a general role in chemotaxis in *Dictyostelium*. The signalling pathway involved in the activation of ERK2 by folic acid has been shown to require the heterotrimeric G protein Gα4 [9], but otherwise has not been well characterized.

In mammalian cells, one signalling pathway for the activation of MAPK involves the upstream activation of the small GTP-binding protein p21ras in response to initial stimulation of a cell surface receptor (reviewed in [10]). Dominant negative versions of p21ras inhibit MAPK activation in a variety of systems and activation of p21ras has been shown to be sufficient to activate MAPK. In this report we describe studies on ERK2 activation in response to cAMP and folic acid in a *Dictyostelium* strain that overexpresses an activated version of RasG (RasG-G12T) [11], the predominant Ras protein expressed during growth and early development. We have measured ERK2 activation by monitoring the tyrosine phosphorylation of the TEY motif, which has been shown to correlate with ERK2 activity in response to both cAMP and folic acid [8,9]. Our results indicate that RasG is a negative regulator of both cAMP- and folic acid-induced ERK2 activation.

The stimulation of the signalling pathways leading to ERK2 activation can be monitored using an antibody specific for the tyrosine-phosphorylated TEY motif of this protein [8]. Tyrosine phosphorylation of ERK2 has been shown to correlate with ERK2 activity as detected by an in-gel kinase assay [8]. Using the antibody assay it was shown previously that folic acid induces a transient phosphorylation of ERK2 in Ax2 cells, peaking at 30–60 s and returning to a barely detectable level by 4 min. The response to folic acid in cells expressing wild-type RasG was similar to that observed previously for Ax2 cells [8], but this response was greatly reduced in cells overexpressing constitutively activated RasG (RasG-G12T) compared to cells overexpressing wild-type RasG (Fig. 1). There was no significant differences in the total amount of ERK2 in the two strains as determined using an anti-ERK antibody indicating that the altered responses were not due to altered levels of ERK2 expression (data not shown).

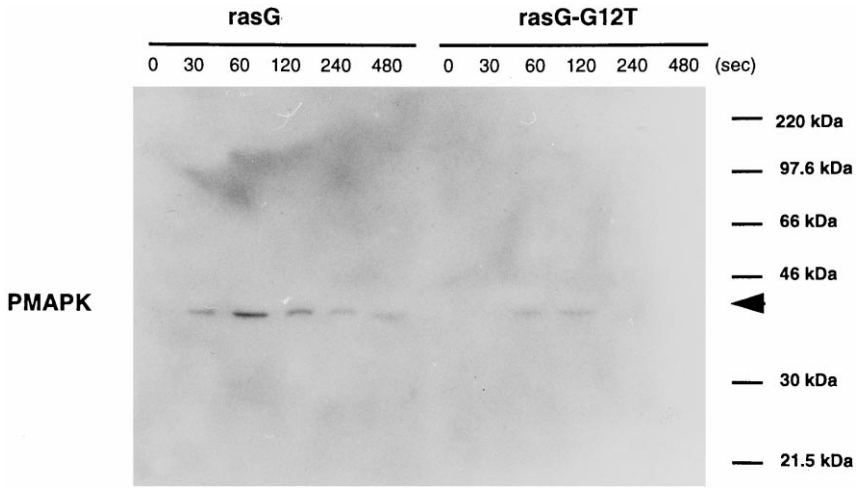

Fig. 1. Folic acid-induced tyrosine phosphorylation of ERK2 in cells overexpressing wild-type or activated RasG. Vegetative cells from strains overexpressing wild-type RasG or activated RasG-G12T [11] were harvested from exponential phase of growth, washed in KK_2 buffer (20 mM potassium phosphate [pH 6.1], 2 mM $MgSO_4$) and resuspended at 2×10^8 cells/ml in KK_2 buffer. Cell suspensions were stimulated with folic acid (100 mM) and lysed by boiling in Laemmli's buffer at the indicated times following folic acid addition. Cell extracts were fractionated by SDS-PAGE, transferred to a membrane and analysed for the presence of phosphorylated ERK2 (PMAPK). As described previously [8], immunoblot analysis to detect phosphorylated MAPK (PMAPK) was performed using PhosphoPlus MAPK antibody kit (New England Biolabs), according to the manufacturer's instructions. The antibody contained in this kit was raised against synthetic phosphotyrosine peptide corresponding to residues 196 to 209 of human p44 MAPK and recognizes MAPK only when catalytically activated by phosphorylation at Tyr 204. All filters were reblotted with anti-ERK antibody (K-23, Santa Cruz Biotechnology) to evaluate total amount of MAPK, and the bound antibodies visualized using ECL–Western blot detection kit (Amersham). The data shown is for a single experiment, representative of four.

The phosphorylation of ERK2 in response to cAMP was monitored in cells which had been starved in shaking suspension for 4 h, with a pulse of exogenous cAMP added every 6 min to induce the cAMP response system. Transformants that overexpress wild-type RasG again showed a response that was very similar to that previously observed in Ax2 cells [8], but ERK2 phosphorylation was significantly reduced in cells overexpressing RasG-G12T compared to cells overexpressing wild-type RasG (Fig. 2). The total level of ERK2 protein was not significantly different in the two strains and was not greatly altered by developing the cells in shaking suspension in the presence or absence of cAMP pulses (data not shown), indicating that the reduced level of ERK2 phosphorylation was not due to an altered level of expression of the ERK2. A small but reproducible increase in cAMP-induced ERK2 phosphorylation was observed in cells overexpressing the dominant negative RasG-S17N (data not shown). These results suggests that RasG acts as an inhibitor of ERK2 phosphorylation in response to cAMP.

There was no significant difference between the degree of ERK2 phosphorylation in response to cAMP in cAMP-pulsed RasG-G12T cells compared to those developed in the absence of pulses (Fig. 3). Since the induction of adenylyl cyclase expression (Fig. 4) and cAR1 expression [11] are enhanced in RasG-G12T-expressing cells that have been pulsed with cAMP compared to those developed in the absence of exogenous pulses, the low level of ERK2 phosphorylation in these cells is not due to a failure to induce the expression of the cAMP response components. It must, therefore, be due to a direct effect of RasG-G12T in a signalling pathway involved in the stimulation of ERK2 activity.

While these experiments were in progress two publications appeared describing work that is directly relevant to our observations. These reports described the influence of a different Ras protein, RasD, on ERK2 activation. In the first of these studies, it was shown that cAMP-induced ERK2 activation, as assessed by an immunoprecipitation kinase assay, was enhanced in strains expressing an activated version of the RasD protein, RasD-G12T [7]. This is similar to the situation in mammalian cells where the activation of p21ras stimulates a signalling pathway that leads to the activation of the highly related MAPKs. In contrast expression of an alternative form of activated RasD (T61Q) inhibited cAMP-induced ERK2 activation, as determined by an in-gel kinase assay [12]. Our results clarify this apparent discrepancy. We have analyzed the tyrosine phosphorylation of ERK2 as a measure of its activation, and find that expres-

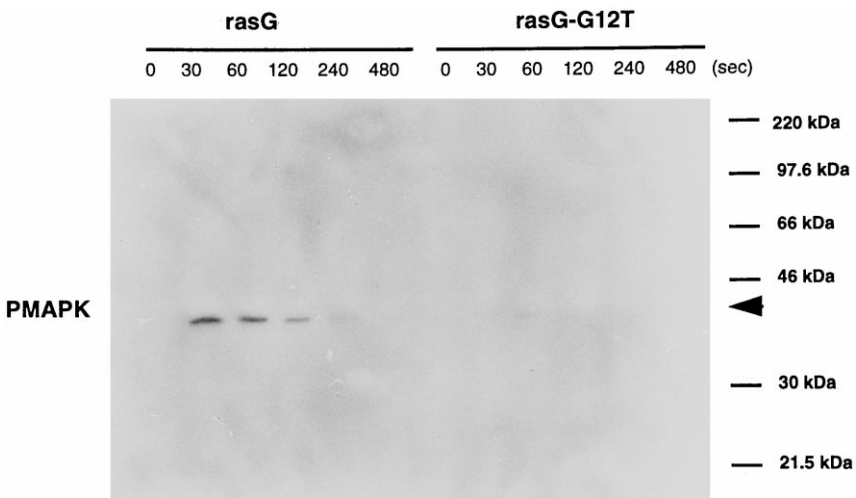

Fig. 2. cAMP-induced tyrosine phosphorylation of ERK2. Strains expressing RasG and RasG-G12T were induced to differentiate by shaking in KK_2 buffer and were pulsed with 25 nM cAMP every 6 min. After washing, cells were resuspended at 2×10^8 cells/ml, stimulated with cAMP (20 μM) and lysed by boiling in the SDS sample buffer at the indicated time. The phosphorylated ERK2 and total ERK2 were detected as described in the legend to Fig. 1. The data shown is for a single experiment, but similar results were obtained in three separate, independent experiments.

sion of activated RasG markedly inhibits ERK2 phosphorylation in response to cAMP, confirming that this assay and the in-gel kinase assay are monitoring the same activation event [8,6]. It is therefore likely that the immunoprecipitation kinase assay that showed a stimulated activation of ERK2 in cells expressing activated RasD [7] is monitoring the activation of a different, related kinase. The fact that RasG and RasD produce the same marked inhibitory response on ERK2 activation in response to cAMP is consistent with the very high level of sequence identity between the two proteins.

It is not clear how activated RasG and RasD negatively regulate this phosphorylation of ERK2. There is no precedent for the idea that Ras proteins may act as negative regulators of signalling pathways; it is therefore more likely that *Dictyostelium* Ras proteins activate a negative regulator of the ERK2 activation pathway. Consistent with this idea is the finding that there is a slight increase in ERK2 phosphorylation in cells expressing a dominant negative version of RasG (RasG-S17N).

The similarity of the reduced ERK2 phosphorylation response to both cAMP and folic acid in the

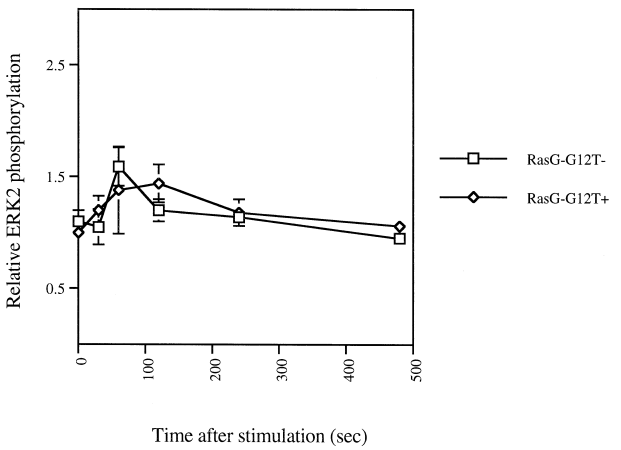

Fig. 3. Comparison of ERK2 phosphorylation in RasG-overexpressing cells developed with and without cAMP pulsing. Cells expressing RasG-G12T were induced to differentiate for the indicated times (in hours) in shaking suspension in KK_2 with (+) or without (−) cAMP pulses (25 nM every 6 min). After washing, cells were stimulated with 20 μM cAMP and lysed at the indicated times as described in Section 2. The amounts of phosphorylated ERK2 relative to total ERK2 were analyzed by Western blotting and quantified by densitometric scanning. The data shown is from a single experiment, representative of two.

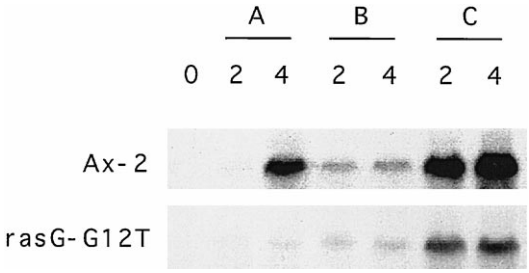

Fig. 4. Expression of *aca* mRNA during early development. Ax2 and RasG-G12T-expressing transformants were induced to differentiate on filters (A), in shaking suspension in MES/PDF (B), or in shaking suspension in MES/PDF, pulsed with 25 nM cAMP every 6 min (C), and were harvested at the indicated times after the initiation of the starvation. Total RNA was analysed by Northern blotting using *aca* cDNA as a probe.

rasG-G12T transformant suggests that RasG is a negative regulator of a step common to both signalling pathways. It is known that both folic acid and cAMP interact with seven transmembrane domain receptors that are coupled to heterotrimeric G proteins (reviewed in Ref. [13]), but it is not known if the two pathways converge prior to the activation of ERK2 or whether they activate ERK2 by parallel but independent pathways. The similarity of the effect of RasG-G12T on the phosphorylation of ERK2 in response to the two chemoattractants suggests that the two pathways do converge. It has previously been demonstrated that constitutive activation of the cAMP-dependent protein kinase (PKA) increases ERK2 phosphorylation in response to cAMP [8,12] but has no effect on the level induced by folic acid [8]. This suggests that PKA can regulate a step in the cAMP-induced pathway which is not similarly regulated in the folic acid induced response. The influence of RasG described here appears to define a common step in the two activation pathways downstream of the step regulated by PKA in the cAMP-induced pathway. The unexpected negative effect of RasG activation on ERK2 phosphorylation suggests that the pathways are unlikely to be identical to those seen in mammalian cells.

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